

## Original Article

## Antioxidant Mechanism of Xiaojin Pill (小金丸) for Treatment of Peyronie's Disease in Rats Based on Matrix Metalloproteinases\*

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**ABSTRACT** **Objective:** To evaluate the effects of Xiaojin Pill (小金丸) in the treatment of Peyronie's disease (PD) in a rat model. **Methods:** Twenty-four male Sprague-Dawley rats were randomly divided into four groups with 6 in each: sham operation, PD model, vehicle control and Xiaojin Pill groups. The rats in the sham operation group received penile tunica albuginea (TA) injection with 50  $\mu$ L vehicle, while the rats in the other 3 groups received 50  $\mu$ L penile TA injection of 50  $\mu$ g transforming growth factor (TGF)- $\beta$  1. Forty-two days after the injection, rats in the vehicle control and Xiaojin Pill groups received 0.5 mL water and Xiaojin Pill solution (107 mg/kg of body weight), respectively by gavage for 28 days, while those in the sham operation and PD model groups did not receive any intervention. After intervention, the expressions of matrix metalloproteinase 2/9 (MMP2/9), nitric oxidesynthase (NOS), superoxide dismutase (SOD) and malondialdehyde (MDA) were measured. **Results:** Rats in the PD model and vehicle control groups presented obvious fibrosis in corpus cavernosum (CC) and demonstrated a significantly increased expressions of MMP2 and MMP9 in the CC compared with the sham operation group (all  $P < 0.01$ ). In contrast, the expressions of MMP2 and MMP9 in the Xiaojin Pill group were significantly down-regulated (both  $P < 0.01$ ). In addition, the levels of NOS and MDA in CC were significantly increased while the activity of SOD was decreased in the PD model and vehicle control groups compared with the sham operation group (all  $P < 0.01$ ). After Xiaojin Pill treatment, the levels of MDA, NOS and SOD appeared to be corrected (all  $P < 0.01$ ). **Conclusions:** Xiaojin Pill could reduce fibrosis in the CC by decreasing the expressions of MMPs, NOS and MDA, and by increasing the activity of SOD. Therefore, Xiaojin Pill might be a therapeutic option for PD.

**KEYWORDS** antioxidant mechanism, Xiaojin Pill, Chinese medicine, Peyronie's disease, rats, matrix metalloproteinases

Peyronie's disease (PD) is a chronic wound-healing disorder characterized by the formation of fibrous inelastic scarring in the tunica albuginea (TA) following trauma to the penis. PD can cause a variety of deformities, including penile hinge defect, curvature, shortening, and narrowing; these deformities affect up to 13% of men.<sup>(1)</sup> Importantly, these deformities significantly disrupt the sexual and psychosocial well-being, causing dyspareunia, and consequently, erectile dysfunction (ED).<sup>(2)</sup> Although the exact etiology of PD is not clear, it is believed that penile repetitive micro-trauma and a subsequent impaired wound-healing process in the connective tissue play an important role in the development of penile plaques and curvature.<sup>(3,4)</sup> In addition, the abundant collagen fibers in the erectile tissue of PD patients may be the result of immune-mediated fibroblast proliferation or a reaction to an infectious agent.

Treatment of PD primarily consists of medication therapy and surgical intervention. Medication therapy includes a diverse range of oral, topical, or intralesional drugs with variable efficacy. Based on the currently available evidence, medications such as vitamin E, potaba, tamoxifen, colchicine, carnitine,

©The Chinese Journal of Integrated Traditional and Western Medicine Press and Springer-Verlag GmbH Germany, part of Springer Nature 2019

\*Supported by the China Postdoctoral Science Foundation (No. 178641)

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DOI: <https://doi.org/10.1007/s11655-019-3203-7>

pentoxifiline, interferon, collagenase, and verapamil only have limited effects in the management of PD.<sup>(5-7)</sup> Surgical intervention can correct penile curvature, which may improve the patient's sexual satisfaction. However, surgery can only be conducted after the acute phase of PD, which may last up to 18 months.<sup>(5)</sup> Due to the risk of progression following surgery, surgical intervention should be postponed for at least 3–6 months after penile deformity and until ED becomes stable. Furthermore, penile pain should ideally be resolved before surgical treatment because pain may be an indicator of active phase and persistent inflammation.<sup>(2)</sup> Recent study has evaluated the effects of electromotive therapy, iontophoresis, radiation therapy, and traction therapy on PD.<sup>(8)</sup> Unfortunately, the majority of approaches are reported to be ineffective or unsuitable for clinical use.

Several studies have explored the pathological features of PD and have found that normal tissue healing restores the baseline levels and organization of extracellular matrix (ECM), whereas fibrosis involving the overgrowth, hardening, and scarring of tissues might be attributed to excess deposition of ECM components.<sup>(9-11)</sup> Moreover, matrix metalloproteinases (MMPs) can adjust the degradation and remodeling of extracellular matrix, so as to promote fibrosis. Meanwhile, MMPs are involved in the process of oxidative stress-induced degradation of endogenous polysaccharide-protein complexes.<sup>(12)</sup> Hence, MMPs may play an important role in oxidative stress.

Xiaojin Pill (小金丸) is a Chinese herbal medicine introduced by the Pharmacopoeia of the People's Republic of China as a potential novel approach to treat internist and surgical diseases.<sup>(13)</sup> The study describing the use of Xiaojin Pill in two patients with PD was reported by Yan, et al<sup>(14)</sup> in 2009. This study concluded that Xiaojin Pill appears to be effective in the treatment of PD. The beneficial effects of Xiaojin Pill have also been reported for other fibrotic diseases.<sup>(15)</sup> Therefore, Xiaojin Pill may represent a promising option for the treatment of PD. Our study aims to investigate the antioxidant effect of Xiaojin Pill in a rat model of PD. Meanwhile we also studied the role of MMPs in the effects of Xiaojin Pill on PD.

## METHODS

### Animals

Twenty-four healthy male Sprague-Dawley rats

(400–450 g License No. SCXK-2011-0011) were generated and group-housed (3–5 per cage) under specific pathogen-free conditions at a constant room temperature of 22–24 °C with a 12-h light/dark cycle (lights on: 8 AM, off: 8 PM). All rats had *ad libitum* access to food (standard rodent diet purchased from Vital River, Beijing, China) and sterile drinking water. Rats used in this study were housed in an animal care facility and the program was accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. This experiment was approved by the Animal Use and Care Committee of Animal Experimentation of The First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, China.

### Grouping, Modeling and Intervention

The rats underwent adaptation to the environment for 7 days. Then, 24 rats were randomized into 4 groups using the random number table method (6 in each), including the sham operation group, PD model group, vehicle group, and Xiaojin Pill group. The treatment protocol for each group was as follows: sham group: 50 μL injection of saline vehicle into the TA and no further treatment for 42 days; PD model group: 50 μg of transforming growth factor (TGF)-β 1 (purchased from PeproTech China, Suzhou, Jiangsu Province, China) mixed with 50 μL vehicle injected into the TA and no further treatment for 42 days;<sup>(16)</sup> vehicle group and Xiaojin Pill group 0.5 mL of water and 0.55 mL Xiaojin Pill solution (107 mg/kg of body weight) were given by gavage twice a day for 28 days beginning 42 days following TGF-β 1 injection. Xiaojin Pill was purchased from Tongrentang, Beijing, China, and was composed of *Moschus*, *Semen Momordicae*, *Radix Aconiti Ferus*, *Resina Liquidambaris*, *Resina Olibanum*, *Commiphora myrrha*, *Faeces Troglodyteri*, *Radix Angelicae sinensis*, *Pheretima*, and Fragrant Ink.

A rat PD model was created using the classic method.<sup>(16)</sup> First, the rats were anesthetized with 10% chloralhydration (0.03 mL/kg) via intraperitoneal injection and were fixed on a surgical plate. A microsyringe was used to vertically penetrate the coronal groove, and was then inserted horizontally about 1 mm. Then, the TA was vertically penetrated. TGF-β 1 was injected along the TA.

Following gavage, the changes in the diet and weight of each rat were assessed and recorded weekly.

Twenty-eight days after intervention, all rats were euthanized by intraperitoneal injection of chloralhydrate (300 mg/kg), and the penile tissues were harvested and stored at  $-80^{\circ}\text{C}$  for further analysis.

### Histology

Haematoxylin and eosin (HE) stain was performed for each penile section with a thickness of 4–6  $\mu\text{m}$  thick. All pathological sections were evaluated by the same experienced pathologist using light microscopy (Leica model DM 2500; Leica Microsystems CMS, Weltzar, Germany). During the process, the pathologist was blinded to rat grouping.

### Western Blot

The penile tissues were homogenized, lysed, and total protein concentration was measured with bicinchoninic acid protein assay kit (Merck Millipore, Germany). Samples containing 40  $\mu\text{g}$  of total protein were loaded and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad, USA). Then, they were transferred to nitrocellulose membrane (0.45  $\mu\text{m}$ ; Millipore, USA), and subsequently, blocked for more than 4 h with 5% bovine serum albumin (BSA)/tris buffered saline buffer. The primary antibodies used for immunoblotting included MMP2/9 (Abcam, Cambridge, USA) and  $\beta$ -non-muscle-actin ( $\beta$ -actin, Santa Cruz Biotechnology, Santa Cruz, USA). All the samples were incubated by primary antibodies at  $4^{\circ}\text{C}$  overnight and then washed by Tris buffered saline Tween (TBST) buffer containing 5% BSA and 0.1% Tween-20. Horseradish peroxidase conjugated goat anti-rabbit was used as the secondary antibody (sc-3836, Santa Cruz Biotechnology) to detect the primary antibodies which was visualized by Gel Imaging System (UVP, California, USA). The bands density was quantified using Image J software (National Institutes of Health, Bethesda, USA) and the average optical density was calculated.

### Biochemical Indices Measurement

Levels of nitric oxide synthase (NOS), SOD and MDA in the penile tissues were determined according to the protocols recommended in the commercial diagnostic kits purchased from Nanjing Jiancheng Institute of Biotechnology Co., Ltd. (Nanjing, China).

### Statistical Analysis

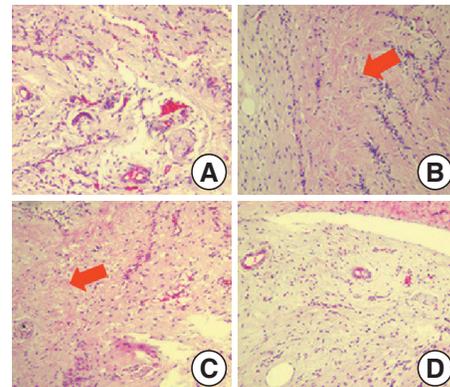
Data were expressed as mean  $\pm$  standard

deviation ( $\bar{x} \pm s$ ). One-way analysis of variance (ANOVA) *post hoc* Dunn's test was used to detect the statistical significance by using SPSS 19.0 software (IBM Co., Armonk, USA). *P* values less than 0.05 were considered significant.

## RESULTS

### Histology

Comparative microscopic evaluation of representative HE staining in penile sections revealed multifocal minimal-to-mild fibrosis, mild-to-moderate fibrosis and more severe attenuation (narrowing) of cavernous spaces in the CC in the PD model and vehicle groups compared with those in the sham operation group. Additionally, rats in the PD model group demonstrated a markedly thickened TA with dense and scattered fibrosis. In contrast, there are no differences in the CC or TA between the PD model and vehicle groups. Compared with the PD model and vehicle groups, the rats in the Xiaojin Pill group presented with a few areas of mild fibrosis along with minimally narrow cavernous spaces in the CC (Figure 1).

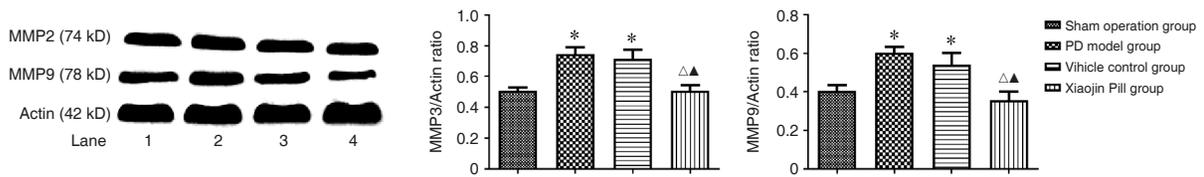


**Figure 1. Histological Assessment of Penile Tissue of Rats by HE Staining ( $\times 100$ )**

Notes: (A) sham operation group: the CC with its normally dilated cavernous spaces surrounded by varied proportions of smooth muscle and fibroelastic connective tissue. (B) PD model group: the presence in the CC of plaques of minimal-to-mild fibrosis and shrunken cavernous spaces in the CC. (C) vehicle control group: the presence of plaques with different degrees of fibrosis and attenuated cavernous spaces in the CC. (D) Xiaojin Pill group: the presence of a few of areas of mild fibrosis along with minimally narrow cavernous spaces in the CC.

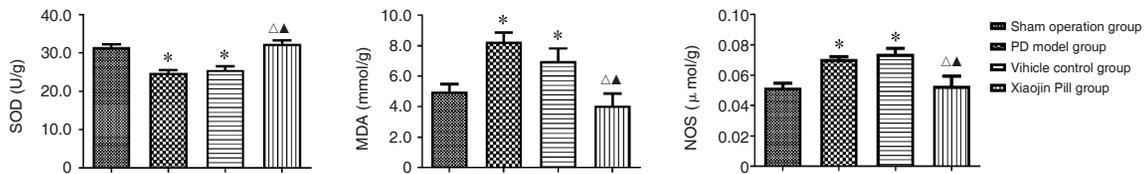
### Effects of Xiaojin Pill on Expressions of MMP2 and MMP9 in Penile Tissue

Rats in the PD model and vehicle control groups demonstrated significantly increased expression of MMP2 and MMP9 in CC compared with the counterparts in the sham operation group (all  $P < 0.01$ ). On the contrary, the expression levels MMP2 and



**Figure 2. Comparison of Total Protein Levels of MMP2/9 in Rat Penile by Western Blot ( $\bar{x} \pm s$ )**

Notes: Lane 1: shame operation group; lane 2: PD model group; lane 3: vehicle group; lane 4: Xiao Pill group. \* $P < 0.01$  vs. the sham operation group; <sup>△△</sup> $P < 0.01$  vs. the PD model group; <sup>△</sup> $P < 0.01$  vs. the vehicle control group;  $n = 6$  in each group



**Figure 3. Comparison of Total Levels of SOD, MDA, and NOS in Rat Penile Tissue**

Notes: The same as Figure 2

MMP9 in the Xiaojin Pill group were significantly down-regulated (all  $P < 0.01$ , Figure 2).

### Effects of Xiaojin Pill on Levels of Oxidation Indices in Rat Penile Tissue

Compared with the sham operation group, the levels of MDA and NOS in CC were significantly increased in the PD model and vehicle control groups (all  $P < 0.01$ ). After treatment with Xiaojin Pill, the levels of MDA and NOS were obviously reduced (both  $P < 0.01$ ). On the other hand, compared with the sham operation group, the activity of SOD was decreased in the PD model and vehicle control groups (both  $P < 0.01$ ). After treatment with Xiaojin Pill, the level of SOD was obviously increased (both  $P < 0.01$ , Figure 3).

## DISCUSSION

PD is a connective tissue disorder of the penile TA and is usually accompanied by ED.<sup>(15)</sup> The TA of the penis plays an important role in the physiology of erections, having an impact on penile elasticity, rigidity, and veno-occlusion.<sup>(15)</sup> Any morphological or functional impairment to the TA can affect the compliance of the penile fibroblastic framework and have profound effects on penile hemodynamic and erectile function. The prevalence of ED in the PD population ranges from 40%–60%. PD is relatively common, affecting nearly 10% of adult men to some degree.<sup>(19)</sup> The therapeutic strategy for PD patients is relatively complicated when considering both the deformity and erectile issues.<sup>(17)</sup> In a pre-experiment, we created a PD rat model via unilateral incision to the TA and demonstrated that Xiaojin Pill can significantly improve cavernosal tissue function and erectile

function. The current study shows that Xiaojin Pill is able to significantly reduce penile TA fibrosis.

PD is a wound-healing disorder that may have similar pathophysiological changes to those that occur with hypertrophic scars.<sup>(17)</sup> It is believed that the dense plaques in PD are due to an imbalance of fibrosis and fibrinolysis. Fibrosis is caused by chronic inflammation resulting from persistent infections, autoimmune reactions, allergic responses, and tissue injuries.<sup>(18)</sup> Unlike the healing process for normal tissue fibrosis, PD involves tissue overgrowth, sclerosis, and scar formation caused by excessive deposition of ECM components. Collagen provides structural and tensile strength in most human tissues and is the most important constituent of ECM.<sup>(19,21)</sup> Fibroblasts and epithelial cells produce MMPs which disrupt the basement membrane and allow inflammatory cells to be recruited to the site of injury.<sup>(22-24)</sup> Chen, et al<sup>(25)</sup> confirmed that *Moschus* can improve immunological liver fibrosis in rats. In our study, we demonstrated that Xiaojin Pill can down-regulate the expression of MMP2/9 and reduce fibrous plaques. Hence, we speculate that Xiaojin Pill can reduce fibrous plaques by regulating the expression of MMP2/9, which inhibits the degradation of ECM. Consequently, we conclude that Xiaojin Pill has antifibrosis properties.

Fibrin can increase plasminogen activator inhibitor 1 (PAI-1) to inhibit fibrinolysis. In addition, fibrin can activate the expression of induced nitric oxide synthase (iNOS) and oxidative stress by increasing reactive oxygen species (ROS). The up-regulated expression of iNOS may affect

wound healing and aggravate fibrosis. Therefore, imbalance of the NO pathway may be a mechanism underlying PD pathophysiology.<sup>(26)</sup> Consequently, the oxidative stress and related fibrogenesis may be the pathogenesis of PD.<sup>(27)</sup> When macrophages and smooth muscle cells are stimulated, the synthesis of NOS is increased. Up-regulation of NOS results in high levels of nitric oxide, which can lead to oxidative stress and vascular relaxation. This process is thought to be involved in the occurrence and development of PD.<sup>(28)</sup> However, one study showed that the level of NOS in CC of PD patients was diminished. Since NOS is necessary for normal penile erection, the decrease in NOS concentration may be a cause of ED in PD patients.<sup>(29)</sup> In our study, we demonstrated that Xiaojin Pill can down-regulate the expression of NOS.

MDA is an end product in lipid peroxidation and reflects the extent of lipid peroxidation while SOD is the main antioxidant enzyme in organisms and has an important role in removing extra free radicals. Xu, et al<sup>(30)</sup> confirmed that *Myrrha* can improve oxidative stress by adjusting ROS. Tan, et al<sup>(31)</sup> reported that *Resina Olibanum* and *Commiphora Myrrha* can improve oxidative stress by adjusting SOD and MDA. Further, Zhang, et al<sup>(32)</sup> demonstrated that *Moschus* can improve oxidative stress by adjusting SOD and MDA. In our study, we demonstrated that Xiaojin Pill can reduce MDA levels and increase SOD levels. Therefore, we assume that Xiaojin Pill can improve antioxidation by regulating the levels of SOD and MDA. Consequently, we conclude that Xiaojin Pill is effective antioxidants.

There are limitations of the present study that should be discussed. First, this study only had a small sample size. Second, the study is limited by the nature of the animal model used; the rat model may not completely address the complexity of the human PD condition. To overcome these limitations, further studies with large sample sizes are needed to assess the effectiveness of Xiaojin Pill in patients with PD.

In summary, our study demonstrated that Xiaojin Pill can reduce fibrosis in the CC by decreasing the expression of MMPs, NOS, and MDA, and by stimulating activity of SOD. Since Xiaojin Pill caused an obvious improvement in the fibrous plaques in PD model rats, we deduce that Xiaojin Pill may have potential therapeutic benefits for men with PD.

The novel properties of Xiaojin Pill provide a new perspective and new potential therapeutic option for the future management of PD.

### Conflict of Interest

There is no conflict of interest.

### Author Contribution

Guo J, Geng Q, and Wang F conceived and designed the study, analyzed the data and wrote the manuscript. OUYANG B, and Han Q designed, performed and analyzed the experiments shown in Figure 1. Chen SF, Li Z, Zhao Y, Gao QH, and Yu GJ designed, performed and analyzed the experiments shown in Figures 2 and 3.

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(Accepted January 25, 2018)  
 Edited by WANG Wei-xia